



Secretion of water soluble pyrroloquinoline quinone glucose dehydrogenase by recombinant *Pichia pastoris*

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Abstract

Secretory production of water soluble pyrroloquinoline quinone glucose dehydrogenase (PQQGDH-B) from *Acinetobacter calcoaceticus* was carried out by using recombinant *Pichia pastoris*. PQQGDH-B is periplasmic protein by processing signal peptide posttranslational process in *A. calcoaceticus*. Instead of the native signal sequence of PQQGDH-B, *Saccharomyces cerevisiae* α -factor signal sequence was used for secretion of PQQGDH-B in *Pichia pastoris* in this study. The productivity of secreted PQQGDH-B achieved 218 kU/liter (43 mg/liter) which is almost the same level as that of recombinant PQQGDH-B previously produced in *E. coli*. The secreted PQQGDH-B in *P. pastoris* was glycosylated but showed similar enzymatic properties as compared with those of recombinant PQQGDH-B produced in *E. coli*. Considering the further optimization of the down stream process and culture condition for high-level production of the recombinant PQQGDH-B by *P. pastoris*, this expression system is expected to achieve industrial level production. © 2002 Elsevier Science Inc. All rights reserved.

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1. Introduction

Glucose dehydrogenases possessing pyrroloquinoline quinone as the prosthetic group (PQQGDHs) are recognized as ideal enzymes for mediator type glucose sensors since the electrochemical cofactor regeneration is not affected by the presence of oxygen in the samples. There are two types of PQQGDH, the membrane-bound single peptide enzyme (PQQGDH-A) and the water-soluble dimeric enzyme (PQQGDH-B). Since PQQGDH-B is not only oxygen independent but also shows a high turnover number, various applications of PQQGDH-B to glucose sensor constituents have been reported [1–7].

The expression of each recombinant PQQGDH-A and PQQGDH-B of *Acinetobacter calcoaceticus* or PQQGDH-A of *Escherichia coli* using *E. coli* as the host strain has been reported by Cleton-Jansen et al. [8–10]. Since both types of PQQGDHs require PQQ and bivalent metal ion for showing enzymatic activities, recombinant PQQGDHs in *E. coli*

were produced as apo enzymes. We previously reported the production of recombinant *E. coli* PQQGDH-A and *A. calcoaceticus* PQQGDH-A using *E. coli* [11–12] or *E. coli* strain harboring heterologous pqq operon from *Klebsiella pneumoniae* to produce holo enzymes in the presence of bivalent metal in the medium [12]. In our study on production of recombinant PQQGDH, we recently reported the recombinant PQQGDH-B production utilizing *K. pneumoniae* as the host strain, and *E. coli* conventional expression vector, which resulted in similar of higher productivity as that of the *E. coli* system, but yielding in holo-enzymes since *K. pneumoniae* is able to synthesis PQQ [13]. However, PQQGDH-B produced in both recombinant systems is being accumulated during the production. Therefore, cell disruption is the essential process for the recovery of these processes. Considering that PQQGDH-B is secreted in the periplasmic space of the Gram-negative bacteria by processing the signal peptide posttranslational process, the extracellular production of recombinant PQQGDH-B will also be expected.

The methylotrophic yeast *Pichia pastoris* is known as a highly successful system for the expression of heterologous genes and successful expressions containing intracellular

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penetration achieved by novel plant proteins. Additions of plant-derived products to animal foodstuffs in the order of 5% of the total fresh mass are already quite common. For example, USDA now allows additions of up to 3.5% of cereal and soy flour or soy concentrate, and up to 2% of soy protein isolates in production of sausages. Consequently, it would not be unrealistic to expect that novel plant proteins could eventually claim at least 25–35% of the fresh mass of ground and processed meats. Recent global consumption has been roughly 40 Mt of ground and processed meat averaging 15% of protein, with an average 20% protein content in mixed (corn and soybean) feed, and with an average 10% conversion rate of plant to animal proteins. Even if suitable novel proteins would eventually supply just 25% of protein content of ground meat and processed meat products this use would lead to a net savings (after taking into account the mass of plant protein that would have to be produced or modified as a substitute) of about 70 Mt of concentrated grain feed.

This would be an equivalent of about 10% of recent annual global consumption of concentrated feeds. Even when using the average US yields of corn and soybeans (both being significantly above the global average) this would mean that more than 15 million hectares of land—an area roughly equal to all farmland in Poland or in South Africa—could be either taken out of production or it could be devoted to other crops. Using lower average corn and soybean yields in Latin America or Asia in order to calculate potential land savings could translate to nearly twice that amount of spared farmland.

As there is a great deal of marginal, erosion-prone land that cannot be sustainably farmed (be it the driest zones of the US Great Plains or North China, both of them now producing feeding corn) its retirement due to the savings obtained by the use of plant proteins would have far-reaching environmental benefits ranging from reduced siltation of streams to reduced eutrophication of waters due to N and P lost from fertilized fields. Alternatively, using the land released from feed production for more sustainable uses, such as grazing lands or orchards, or planting it to bioengineered crops producing high levels of micronutrient, would have different, but no less important environmental benefits. And, naturally, reduced feeding of animals would result in lower output of wastes and reduced losses of nutrients to water and to the atmosphere, and less methane.

In order to attain truly sustainable agricultures we will have to rethink and reform most of the practices that prevail today. Their ideal performance should be determined by moving backwards along the production chain, starting with maximum acceptable amounts of nitrate leaching from fertilizers or ammonia volatilization from animal wastes and establishing allowable long-term tolerances of soil erosion and reliable supplies of irrigation water. Only after establishing these rates, and other key environmental parameters, we should determine, given our best practices and technical possibilities, what crops could be grown and what yields

should be targeted. Meat production based on concentrate feeding would then emerge as a residue of feedstuffs (plus, obviously, all suitable food processing residues) that could be produced sustainably on land not needed for securing essential food crop requirements.

Before we reach this optimal state of truly environmentally driven agriculture we must explore every opportunity for increased efficiency of entire food chains. Reduced nutrient and water losses during fertilization and irrigation are the most obvious ingredients of such a strategy—but using plant-derived proteins in order to moderate the environmental impacts of meat production should become a major part of this effort as it offers substantial long-term payoffs, benefiting both ecosystems and human health.

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expression or secretory expression have been reported [14–16]. One of the important factors for this system is alcohol oxidase I (AOX1) promoter of *P. pastoris* which can control the expression of foreign genes by the concentration of methanol. Furthermore, since the molecular genetic manipulation of *P. pastoris* is similar to that of *Saccharomyces cerevisiae* of which the experimental system is well-characterized, the expression system by *P. pastoris* is also widely accepted.

Here, we report the secretory production of recombinant PQQGDH-B using *P. pastoris* as the host strain and its over-expression system utilizing alcohol oxidase promoter, combined with the *Saccharomyces cerevisiae* α -factor secretion system.

2. Materials and methods

2.1. Bacterial strains and plasmid construction for secretion of PQQGDH-B

Plasmid pGB containing *A. calcoaceticus* soluble PQQGDH [17] was used as a template, and PCR was performed with the following primers designed with restriction enzyme sites.

5'-GGCTCGAGAAAAGAGATGTTCTCTAACTCCAT-CTCAA-3'

Xho I site

5'-GGGAATTCTTACTTAGCCTTATAGGTGAACCTTA-TGATAT-3'

Eco RI site

PCR products were digested with *Xho* I and *Eco* RI. Since another *Xho* I site exists in internal gene, two fragments were obtained. One fragment, *Xho* I–*Eco* RI, was inserted into the *Xho* I and *Eco* RI sites of *P. pastoris* secretion vector pPIC9 (Invitrogen, Carlsbad, CA) and digested with *Xho* I. As the next step, the other fragment, the *Xho* I–*Xho* I fragment, was inserted into the prepared vector digested with *Xho* I. The constructed vector pPIC9GB2 contains complete PQQGDH-B structural gene not under the original PQQGDH-B signal sequence but under α -factor signal sequence (Fig. 1).

2.2 Expression of PQQGDH-B in *P. pastoris*

The constructed plasmid pPIC9GB2 was digested by *Sal* I to be linearized. 5 μ g of the linearized pPIC9GB2 was used for electroporation of *P. pastoris* host strain K_m71 (MutS, *his4*, *aox1::ARG4*) from Invitrogen (Carlsbad, CA), using electroporator GenePulser (Bio-Rad, CA, SA). Using the MD (1.34% yeast nitrogen base (YNB), 1.61 μ M biotin, 2% dextrose) agar plate, the recombinant *P. pastoris* was selected after incubation for 48–60 h at 28°C (His⁺ selection).

A single colony of transformants was grown with 100 ml of BMGY (1% yeast extract, 2% peptone, 100 mM potas-

sium phosphate, pH 6.0, 1.34% YNB, 1.61 μ M biotin, 1% glycerol) in 500 ml of shaking flask until O.D.₆₀₀ = 5 at 30°C. The grown culture was centrifuged and the pellet was suspended with 20 ml of BMMY (identical to BMGY except for 0.5% methanol instead of 1% glycerol). The resuspended culture was grown for 120 h by addition of 0.5% methanol every 24 h. Since PQQGDH-B was designed under alcohol oxidase promoter, the expression is induced by methanol.

2.3. Purification of secreted PQQGDH-B

The expressed recombinant PQQGDH-B in the medium supernatant was subjected to an ammonium sulfate precipitation (70%) after removing the cells by centrifugation at 4°C, 3,000 \times g for 10 min. The suspension was subjected to ultra-centrifugation (160,500 \times g, 30 min, 4°C), and the pellet, which was formed by ammonium persulfate precipitation, was re-suspended in 20 ml of 10 mM potassium phosphate buffer, pH 7.0 and dialyzed against the same buffer twice. The protein extract was stirred in 20 ml of CM-Toyopearl 650 M cation exchange resin (Tosoh, Japan) equilibrated with 10 mM potassium phosphate buffer, pH 7.0. The protein was eluted with 5 ml of 1M NaCl in a 10 mM potassium phosphate buffer, pH 7.0. The eluted solution was dialyzed overnight against a 10 mM potassium phosphate buffer, pH 7.0. and stirred in 5 ml of DEAE-Toyopearl 650M anion exchange resin (Tosoh, Japan) with a 10 mM potassium phosphate buffer, pH 7.0. The resin was loaded to an open column and the protein was eluted 5 ml of 1.0 M NaCl in a 10 mM potassium phosphate buffer, pH 7.0. These chromatographies were carried out batchwise at 4°C. The purified enzyme, found to be electrophoretically homogeneous by silver staining on SDS-PAGE, was utilized for the following analyses. The total protein concentration was determined using DC protein Assay Kit (Bio-Rad, CA, USA). Each enzyme sample was incubated in a 10 mM MOPS buffer, pH 7.0 with 1 mM CaCl₂ and 1 μ M PQQ for 30 min at room temperature for GDH assay. The enzyme activity was determined in the presence of 0.6 mM phenazine methosulfate, 0.06 mM 2,6-dichlorophenolindophenol (DCIP) at 25°C, and a 50 mM glucose concentration, by measuring the decrease in absorption of DCIP at 600 nm.

2.4. Characterization of recombinant PQQGDH-B

The recombinant PQQGDH-B secreted from *P. pastoris* was used for the N-terminal amino acid sequence analysis by PPSQ-10 (Shimadzu, Japan).

The enzymatic activities of secreted PQQGDH-B in *P. pastoris* toward each substrate were obtained at 20 mM, and were compared with the activity for 20 mM glucose as a control, as previously described [18]. Each experiment was repeated for 8 times.

Kinetic parameters of secreted PQQGDH-B in *P. pasto-*

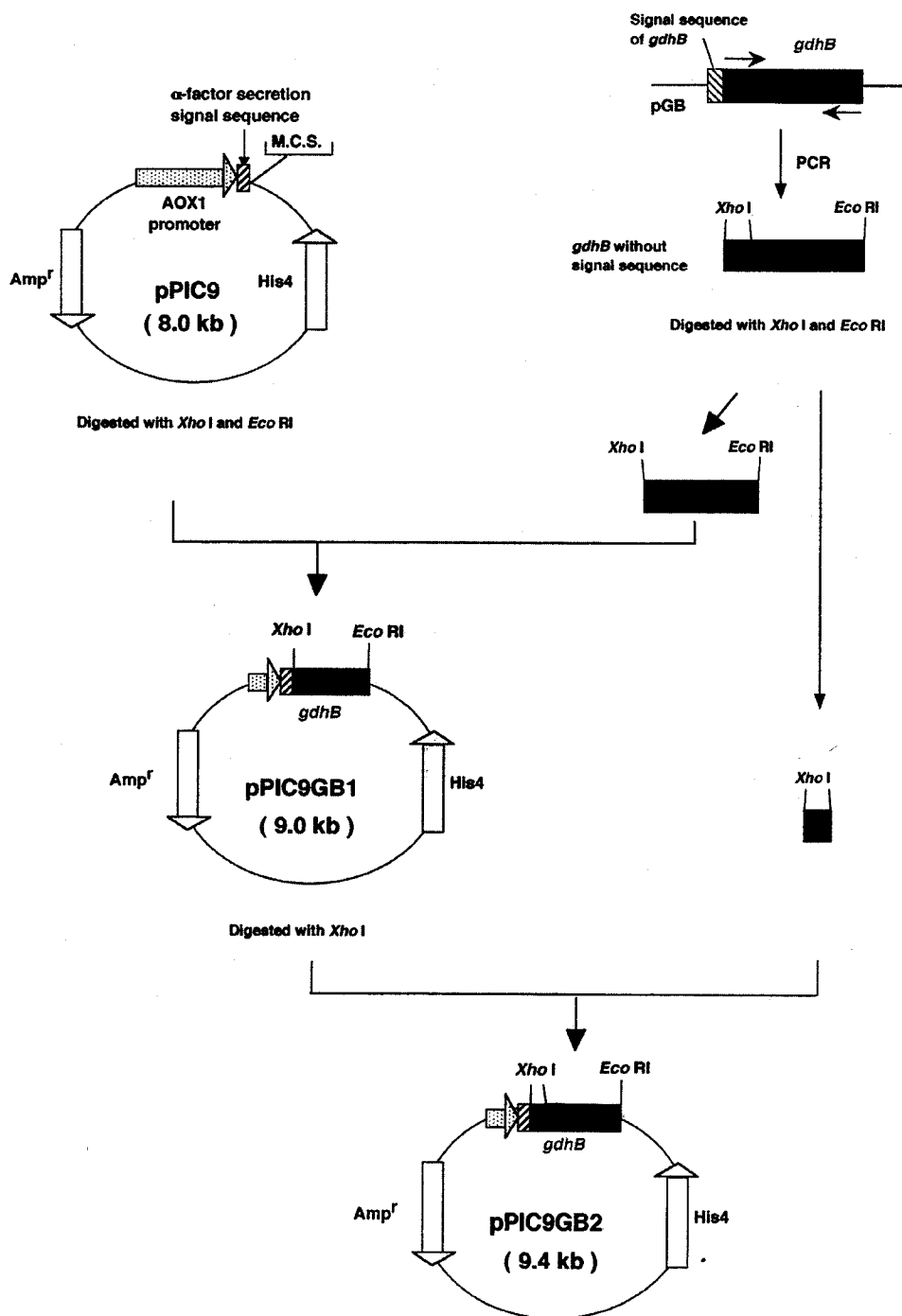


Fig. 1. Construction of plasmid pPIC9GB2 for the expression of PQQGDH-B in *P. pastoris*.

ris for glucose and lactose were determined by measuring of enzymatic activity as described in materials and methods 3, but utilizing various concentration of each substrate. The kinetic parameters of secreted PQQGDH-B were compared with those of PQQGDH-B produced in *E. coli*.

EDTA tolerance of secreted PQQGDH-B in *P. pastoris* was determined by measuring the residual activities of the

samples which were taken periodically from incubation in 10 mM MOPS-NaOH (pH 7.0) containing 5 mM EDTA.

The thermal stability of secreted PQQGDH-B was determined using 19 μ g/mL purified enzyme. The time course of thermal inactivation at 55°C was measured as previously described [17] and was compared with that of purified PQQGDH-B produced in *E. coli*.

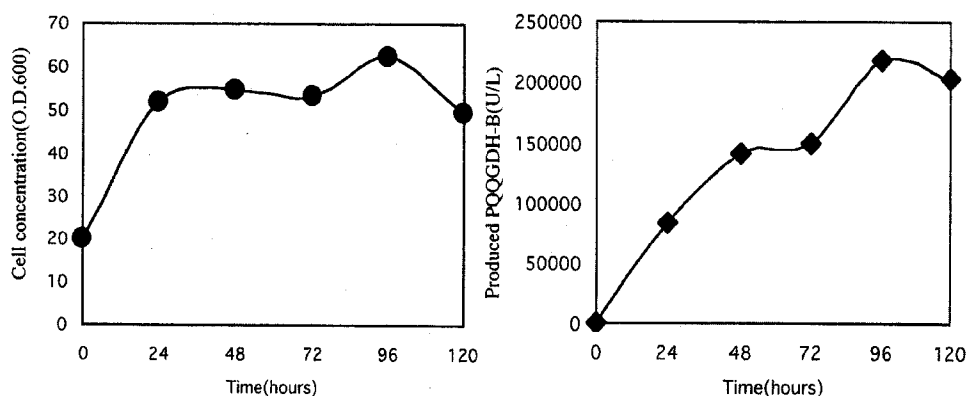


Fig. 2. Time course of cell growth and produced recombinant PQQGDH-B. ●; cell concentration O.D.600 ◆; Produced PQQGDH-B [U/liter].

Purified enzyme was deglycosylated by incubating 50 μ g of total protein with 2.4 U N-glycosidase F for 1 h at 37°C according to the instruction manual of N-glycosidase F deglycosylation kit (Roche Diagnostics GmbH, Germany).

The quantitative analysis of glycosylation of secreted PQQGDH-B was carried out by measuring neutral sugar after deglycosylation procedure. The neutral sugar was analyzed by phenol-sulfuric acid method using mannose as the standard.

3. Results

3.1. Production and purification of secreted PQQGDH-B in *P. pastoris*

Fig. 2 shows the time course of cell growth and produced PQQGDH-B in *P. pastoris*. Cell concentration of recombinant *P. pastoris* rapidly increased up to O.D.600 = 70 within 24 h cultivation and was stable until the 120 h point. The produced GDH from recombinant *P. pastoris* in the medium also increased according to the cell growth and reached 218 kU/liter within 96 h cultivation. The purification of recombinant PQQGDH-B was carried out using an 80 ml culture supernatant. The results of each purification step are presented in Table 1. The specific activity after the

final step was 5080 U/mg-protein. On the basis of this specific activity, the productivity of this enzyme was calculated to be 43 mg/liter culture. The yield was 38.5%.

3.2. Enzymatic properties of secreted PQQGDH-B

The 10 amino-terminal residues of the secreted PQQGDH-B were identical to the native PQQGDH-B, Asp-Val-Pro-Leu-Thr-Pro-Ser-Gln-Phe-Ala. This implies correct recognition and processing of the α -factor signal sequence.

Fig. 3 shows the SDS-PAGE of secreted PQQGDH-B before and after N-glycosidase F treatment for the deglycosylation of putative glycosyl-moiety. After N-glycosidase F treatment of secreted PQQGDH-B in *P. pastoris*, a single major band of the same size as native PQQGDH-B, 50 kDa

Table 1
Purification of recombinant PQQGDH-B secreted in *P. pastoris*

	Total activity [U]	Total protein [mg]	Specific activity [U/mg protein]	Yield [%]
Culture supernatant	16000	(898)*	17.8	100
Ammonium sulfate precipitation	14600	70.5	207	91.2
CM Toyopearl	11400	6.8	1670	71.3
DEAE Toyopearl	6200	1.2	5080	38.5

The purification of recombinant PQQGDH-B was carried out using 80 mL culture supernatant.

* The total protein in the culture supernatant contained the medium component, which was about 800 mg (10 g/L) in the initial condition.

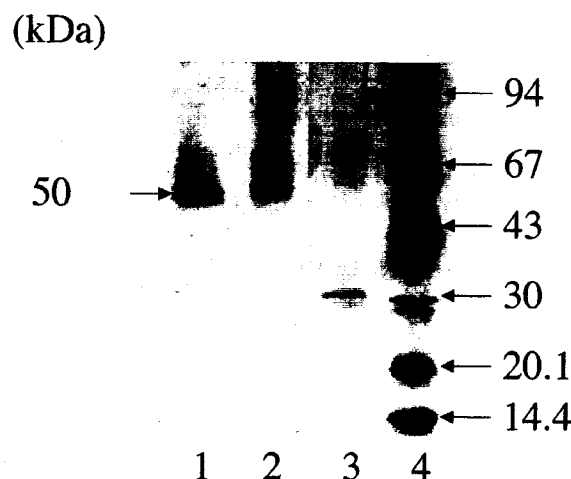


Fig. 3. SDS-PAGE of secreted PQQGDH-B. The samples before and after deglycosylation with N-glycosidase F were subjected to SDS-PAGE. Arrows show (a) glycosylated PQQGDH-B, (b) deglycosylated PQQGDH-B and (c) deglycosylating enzyme N-glycosidase F. Samples: 1, secreted PQQGDH-B treated with N-glycosidase F; 2, Secreted PQQGDH-B; 3, N-glycosidase F; 4, LMW standard.

Table 2
Substrate specificity of recombinant PQQGDH-B produced in *E. coli* and secreted in *P. pastoris*

Substrate	PQQGDH-B produced in <i>E. coli</i> (\pm): standard deviation	PQQGDH-B secreted in <i>P. pastoris</i> (\pm): standard deviation
Glucose	100% (1.86)	100% (5.13)
3-o-m-D-glucose	75.4% (1.56)	72.1% (2.77)
Allose	34.1% (1.17)	32.0% (1.17)
Galactose	15.6% (1.15)	13.8% (1.41)
Maltose	73.5% (2.38)	83.6% (2.47)
Lactose	56.9% (1.92)	59.9% (1.64)
Cellobiose	91.0% (2.65)	100% (3.18)
Xylose	12.8% (1.00)	11.2% (0.86)
2-Deoxy-D-glucose	6.48% (0.55)	7.01% (0.83)
Mannose	10.5% (1.75)	13.2% (2.01)

Each enzyme activity was measured at 20 mM substrate concentration and compared with glucose as the control. Each experiment was repeated for 8 times and SD was calculated.

was observed in SDS-PAGE. Considering the sample before N-glycosidase F treatment did not show a discrete band but rather a smear in the range of 50–70 kDa in the SDS gel, secreted PQQGDH-B in *P. pastoris* was suggested to be glycosylated with high mannose-type oligosaccharides of varying grade of polymerization.

In the analysis of the oligosaccharide content in secreted PQQGDH-B, 1.8 μ g of mannose was estimated as concerned as posttranslational modification, glycosylation. The amount of oligosaccharide corresponds to 36.8% of the secreted PQQGDH-B.

The substrate specificity of PQQGDH-B produced in *E. coli* and *P. pastoris* was compared in Table 2. Each relative activity for substrates of PQQGDH-B secreted in *P. pastoris* showed similar value to each relative activity for substrates of PQQGDH-B produced in *E. coli*.

The K_m value to glucose and lactose of secreted PQQGDH-B was the same, 17.5 mM, and the V_{max} value was 5000 U/mg-protein to glucose and 2800 U/mg-protein to lactose (Table 3). The K_m value to lactose of secreted PQQGDH-B in *P. pastoris* did not show a big difference from that of PQQGDH-B produced in *E. coli*, and the V_{max} value to lactose of secreted PQQGDH-B was 1.4 times higher than that of PQQGDH-B produced in *E. coli*.

Table 3
Kinetic parameters of recombinant PQQGDH-B produced in *E. coli* and secreted in *P. pastoris*

	Glucose		Lactose	
	Km [mM]	Vmax [U/mg protein]	Km [mM]	Vmax [U/mg protein]
PQQGDH-B produced in <i>E. coli</i>	25.0	4610	18.9	1982
PQQGDH-B secreted in <i>P. pastoris</i>	17.5	5000	17.5	2800

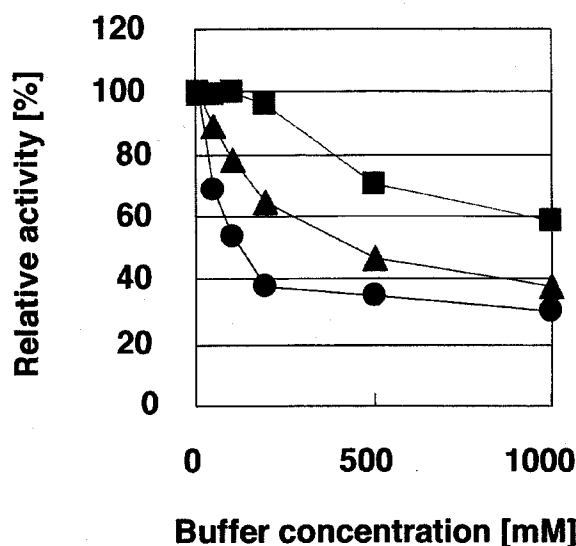


Fig. 4. The effect of concentration of buffer for recombinant PQQGDH-B produced in *E. coli*, glycosylated PQQGDH-B in *P. pastoris* and deglycosylated PQQGDH-B.

●; PQQGDH-B produced in *E. coli*
■; PQQGDH-B secreted in *P. pastoris*
▲; Deglycosylated PQQGDH-B by N-glycosidase F

Each enzymatic activity for the samples (recombinant PQQGDH-B produced in *E. coli*, glycosylated PQQGDH-B secreted in *P. pastoris* and its deglycosylated PQQGDH-B by N-glycosidase F) was measured using various concentrations of buffer and the enzymatic activity at 0 mM was defined as the respective initial activity.

The EDTA tolerance and the thermal stability of PQQGDH-Bs produced either in *P. pastoris* or *E. coli* were examined. The EDTA tolerance is the indication of their stabilities in co-factor binding. Both PQQGDH-Bs maintained high residual activities and showed over 90% of each initial activity after 10 min incubation in the presence of 5 mM of EDTA, therefore, *P. pastoris* secreted enzyme retained similar co-factor binding stability as that of produced in *E. coli*. The thermal stability of both PQQGDH-B produced in *P. pastoris* and *E. coli* showed similar profile at 55°C. After 10 min incubation at 55°C, both of the residual activities were around 50%.

These results indicate that the secreted PQQGDH-B from *P. pastoris* has almost identical enzymatic properties even though it was glycosylated.

We then investigated the enzymatic activity of glycosylated PQQGDH-B in various concentration of MOPS-NaOH buffer, from 10 to 1000 mM to see the effect of ionic strength on enzymatic activity. In the buffer concentration range we tested glycosylated PQQGDH-B showed higher relative activity compared with other PQQGDH-B samples, produced in *E. coli* and deglycosylated by N-glycosidase F (Fig. 4). It showed 70% of initial activity at 500 mM and 60% of initial activity at 1000 mM. Whereas the relative activities of recombinant PQQGDH-B produced in *E. coli* at 500 mM and 1000 mM were less than 40%. Deglycosylated PQQGDH-B showed intermediate values of

relative activity for all tested concentrations between glycosylated PQQGDH-B and recombinant PQQGDH-B in *E. coli*, though the relative activity of deglycosylated PQQGDH-B is a rather similar to that of native PQQGDH-B. Therefore, the glycosylated PQQGDH-B was not sensitive to the alteration in buffer ionic strength.

4. Discussion

Recombinant PQQGDH-B was successfully secreted by *P. pastoris* in this study and the productivity of recombinant PQQGDH-B was 43 mg/liter. Considering some reports of secretory expression in *P. pastoris* at the level of over 1 g/liter or few reports of over 10 g/liter secretory production, the productivity in this study is not so high level. The volumetric productivity of secretory production of PQQGDH-B achieved 218 kU/liter which is an almost similar value to the cell extract achieved by the *E. coli* expression system (193 kU/liter).

In order to increase the secretory productivity of recombinant PQQGDH-B in *P. pastoris*, further optimization of culture condition and purification steps are required. Ohashi et al. reported the perfusion culture technique using a shaken ceramic membrane flask for high level secretory production of human serum albumin by *P. pastoris* [19]. The higher productivity of recombinant secreted PQQGDH-B will be also achieved by keeping high-expression active cells by keeping sufficient DO level with keeping high cell density. Meanwhile, the optimization of medium will also enhance the productivity of secretory production of recombinant enzyme. Increasing in the yield of the mouse epidermal growth factor was observed by the addition of 1% casamino acids and buffering the medium to pH 6.0 [20]. The investigation of the alternative expression system may also improve the expression level. Paifer et al. has reported the construction of cloning vectors using the signal sequence of the alpha amylase gene and the SUC2 gene signal sequence from *Saccharomyces cerevisiae* to express *Bacillus licheniformis* alpha amylase in *P. pastoris* [16]. Although the secretory level of PQQGDH-B in this system was not in the same levels for that achieved for the secretory production of high-eucaryotic proteins, the further optimization for down-stream processing will lead to industrialize yeast secretory production of PQQGDH-B, considering that the level of the expression in this system is similar with that of recombinant PQQGDH-B production in *E. coli*.

The secreted PQQGDH-B in *P. pastoris* was glycosylated, which is confirmed from the results of SDS-PAGE after N-glycosidase F treatment of secreted PQQGDH-B and quantification of oligosaccharide content in secreted PQQGDH-B by the colorimetric phenol-sulfuric acid method. During the chromatographic procedure for secreted PQQGDH-B, the enzyme adsorbed on both CM(cation exchange) and DEAE(anion exchange) resins, in the buffer with similar pKa. However, these behavior were not due to

the result of glycosilation but it is inherent property of this enzyme. The native PQQGDH-B produced in *Acinetobacter calcoaceticus* is also adsorbed in both resins in the buffers with similar pKa [21,22]. In the analysis of the effect of buffer concentration, deglycosylated PQQGDH-B showed a intermediate profile between those of glycosylated PQQGDH-B and recombinant PQQGDH-B produced in *E. coli*. Considering the secreted PQQGDH-B in *P. pastoris* was supposed to be glycosylated with high mannose-type oligosaccharides of varying grades of polymerization, deglycosylation procedure of the recombinant PQQGDH-B secreted in *P. pastoris* by N-glycosidase F might not yield in the complete homogeneous de-glycosylated enzyme sample, but contain various grade of insufficiently deglycosylated PQQGDH-B. Such mixture of various deglycosylated PQQGDH-B possibly shows the intermediate profile between that of glycosylated and completely deglycosylated PQQGDH-B in the investigation of the ionic strength dependency.

Although the secreted PQQGDH-B in *P. pastoris* was glycosylated, the glycosylated PQQGDH-B showed almost identical enzymatic properties compared with those of recombinant PQQGDH-B in *E. coli* except that glycosylated enzyme was not significantly affected by the change in buffer ionic strength. Further investigation of glycosilation site is essential to elucidate whether the phenomenon is specific for glycosilation sites or is dependent on that the fact that the enzyme is surrounded by oligo-saccharide and is dependent on the microenvironmental change.

Although, the further optimization of the expression and cultivation of the recombinant PQQGDH-B by *P. pastoris* is required, this expression system of PQQGDH-B is expected to be a powerful tool in industrial level production of this enzyme.

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